MOLECULAR REPRODUCTION AND DEVELOPMENT 56:113–123 (2000)

DNA Microarray Analyses of Genes Regulated During the Differentiation of Embryonic Stem Cells

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ABSTRACT Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts, and in response to retinoic acid (RA) are induced to differentiate to form some of the first distinguishable cell types of early mammalian development. This makes ES cells an attractive model system for studying the initial developmental decisions that occur during embryogenesis and the molecular genetics and associated mechanisms underlying these decisions. Additionally, ES cells are of significant interest to those characterizing various gene functions utilizing transgenic and gene-targeting techniques. With the advent of DNA microarray technology, which allows for the study of expression patterns of a large number of genes simultaneously within a cell type, there is an efficient means of gaining critical insights to the expression, regulation, and function of genes involved in mammalian development for which information is not currently available. To this end, we have utilized Clontech's Atlas Mouse cDNA Expression Arrays to examine the expression of 588 known regulatory genes in D3 ES cells and their RA-induced differentiated progeny. We report that nearly 50% of the regulatory genes are expressed in D3 and/or D3-differentiated cells. Of these genes, the steady-state levels of 18 are down-regulated and 61 are up-regulated by a factor of 2.5-fold or greater. These changes in gene expression are highly reproducible and represent changes in the expression of a variety of molecular markers, including: transcription factors, growth factors and their receptors, cytoskeletal and extracellular matrix proteins, cell surface antigens, and intracellular signal transduction modulators and effectors. *Mol. Reprod. Dev. 56:113–123, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: cDNA microarray; embryonic stem cells; retinoic acid; mammalian embryogenesis; development; differentiation

INTRODUCTION

Large-scale study of gene expression is a hallmark of the transition from "structural" to "functional" genomics, where knowing the complete sequence of the genome is only the first step in understanding how specific genes function. The next and more challenging step is the characterization of the biological roles of genes and the manner in which their encoded proteins carry out cellular processes. Central to understanding these roles is to define the gene expression profile of a large number of genes under a wide range of conditions in a sensitive, quantitative, and efficient fashion. To this end, a promising approach for simultaneously analyzing the expression pattern of multiple genes is the hybridization of cDNA probes synthesized from mRNA populations prepared from cells and/or tissues to nucleic acid arrays (Lennon and Lehrach, 1991; Gress et al., 1992; Fodor et al., 1993; Schena et al., 1995). This technology, otherwise known as cDNA microarray expression profiling, offers tremendous potential for characterizing gene expression patterns during normal biological and disease processes, as well as for the identification of differentially expressed genes that may play an integral role in these processes. In this regard, using this approach has resulted in the identification and cloning of genes with potential relevance to growth control and terminal differentiation in human melanoma cells (DeRisi et al., 1996; Huang et al., 1999), ovarian carcinomas (Wang et al., 1999), renal cell carcinoma (Moch et al., 1999), glioblastomas (Sehgal et al., 1998; Fuller et al., 1999; Rhee et al., 1999) and breast cancer (Hoch et al., 1999). In addition, cDNA microarrays have been used to study the temporal program of gene expression in human fibroblasts in response to serum (Iyer et al., 1999) as well as the genome-wide expression pattern of genes in yeast (Wodicka et al., 1997).

Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts and retain their pluripotency during in vitro culture (Evans and Kaufman, 1981; Martin, 1981). In response to exposure to retinoic acid (RA), ES cells are induced to differentiate to form many of the first distinguishable cell types of the early mammalian embryo, including the extraembryonic endoderm (Evans and Kaufman, 1981; Martin, 1981).

Grant sponsor: National Institutes of Health; Grant number: CA 74771, Grant sponsor: National Cancer Institute; Grant number: CA 36727.

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Received 10 October 1999; Accepted 9 November 1999

This makes ES cells a particularly attractive model system for studying the initial developmental decisions that occur during embryogenesis and the molecular genetics and associated mechanisms underlying these decisions. However, the majority of studies devoted to identifying biologically relevant, differentially expressed genes between ES cells and their differentiated progeny have been limited to the quantitation of expression of at most a few genes at a time. The use of microarray technology enables one to monitor the expression of a large number of genes simultaneously and provides the means for gaining critical insights into the expression, regulation, and function of genes involved in mammalian development for which information is currently not available. In addition, ES cells are of significant interest to those characterizing various gene functions utilizing transgenic and gene-targeting techniques (Wilder and Rizzino, 1993). Comparing the expression patterns of a myriad of genes for both wild-type and genetically altered ES cells will enhance considerably the ability to identify and characterize biological roles for developmentally important genes. In the present study, we have utilized Clontech's Atlas Mouse cDNA Expression Arrays to examine the expression of 588 known regulatory genes in D3 ES cells and their RA-induced differentiated progeny. We report that nearly 50% of the regulatory genes are expressed in D3 ES and/or D3-differentiated cells. Of these genes, the steady-state levels of 18 are down-regulated and 61 are up-regulated by a factor of 2.5-fold or greater.

MATERIALS AND METHODS

Growth and Differentiation of ES Cells

D3 ES cells were cultured on gelatin-coated flasks in a high-glucose-containing DME medium (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), and 10 ng/ml recombinant human leukemia inhibitory factor (LIF; Pepro Tech, Inc., Rocky Hills, NJ), as described previously (Ma et al., 1992). Differentiation of ES cells was induced by the addition of 1 μM all-*trans*-retinoic acid (RA; Fisher Scientific, Pittsburgh, PA) to culture medium containing 2 ng/ml LIF. ES cells undergoing differentiation were refed with fresh medium every 24 hr. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

RNA Isolation

A FastTrack 2.0 kit (Invitrogen, San Diego, CA) was used to isolate poly(A)$^+$ RNA from nearly confluent cultures of D3 ES cells and D3 ES cells induced to differentiate with RA for 96 hr. After confirmation of the integrity of the mRNA on an agarose gel, 10 μg aliquots of mRNA were treated with one unit of RNase-free DNase I (Boehringer Mannheim Corporation, Indianapolis, IN) to eliminate potential genomic DNA contamination.

cDNA Array Hybridization

Broad-scale expression profiling between D3 ES cells and D3-differentiated cells was performed using Atlas Mouse cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA), a technique whose basic principle can be viewed as reverse Northern blotting. Each array is a positively charged nylon membrane (8 × 12 cm) that is spotted in duplicate with cDNA fragments representing 588 known genes and 21 housekeeping genes or control sequences. Each cDNA fragment is 200–600 bp long and has been amplified from a region of the transcript that lacks the poly-A tail, repetitive elements, or other highly homologous sequences to minimize cross hybridization and the nonspecific bindings of cDNA probe. Genes are arrayed in six quadrants with genes of like function (i.e., oncogenes, transcription factors, assorted receptors, etc.) grouped together geographically. A list of these genes, including array coordinates and GenBank accession numbers, is available at Clontech's Web site (www.clontech.com/archive/JAN98UPD/Atlaslist.html). For side-by-side array hybridizations, 1 μg of mRNA from each cell population was reverse-transcribed using [α-^{32}P]dATP (10 μCi/μl, 3,000 Ci/mmol; Amersham Pharmacia Biotech; Arlington Heights, IL) and the reagents provided in the Atlas cDNA Expression Array kit to synthesize ^{32}P-radiolabeled cDNA probes. Radio-labeled probes were denatured under basic conditions, neutralized, and then added to separate 5 ml aliquots of ExpressHyb hybridization solution (Clontech) containing 100 μg/ml heat-denatured sheared salmon testes DNA (Sigma) to reach final probe concentrations of approximately 7 × 10^5 cpm/ml. Hybridization/cDNA probe solutions were applied to prehybridized Atlas Arrays (1 hr in ExpressHyb/salmon testes DNA at 68°C in the absence of a labeled probe) and hybridized overnight at 68°C. After hybridization, membranes were washed twice with 200 ml of 2× SSC, 1% SDS solution at 68°C for 30 min, followed by two 30-min washes in 200 ml of 0.1× SSC, 0.5% SDS, at 68°C. Then the membranes were rinsed in 0.1× SSC and exposed to phosphor screens for various lengths of time to ensure linear comparison ranges for both strongly and weakly expressed genes.

Quantitation of Gene Expression

Hybridized Atlas Arrays were visualized and quantitated by PhosphoImager (Molecular Dynamics Inc., Sunnyvale, CA) at a pixel resolution size of 88 microns. Autoradiographic intensity was analyzed using Analytical Imaging Station (AIS) software (version 3.0 r.1.3; Imaging Research Inc., St. Catharines, Ontario, Canada). A grid matrix was generated and applied to the phosphorimage of each Atlas Array, which identified the duplicated target location for each of the 588 known genes as well as the 21 housekeeping genes and control sequences. The intensity of hybridization signals for each target location was determined automatically and corrected for background using the intensity values of pixels surrounding the target areas. Calculated intensi-

ties correlate linearly with the concentration of target mRNAs present in the total mRNA population, since the amount of target cDNA attached to the membrane was in excess (10 ng) and the background hybridization signals were sufficiently low. For assessing differences in gene-expression between arrays (mRNA populations), the intensity value of each known gene was normalized in two different ways: to the target intensity values of designated housekeeping genes and to the sum of the intensity values of all of the genes. We found little difference in the normalization coefficients generated by these two methods. Comparison between mRNA populations from parental D3 ES cells and their RA-induced differentiated progeny was performed with four different sets of Atlas Array membrane lots. Only those genes which showed an average fold induction or reduction of 2.5-fold or greater across all four membrane lots were listed as differentially expressed. Correlations and differences in gene expression between mRNA populations were also compared by scatter plot analysis, in which each point represents a particular gene and the location of the point is determined after normalization by the intensity value of the gene in the mRNA population represented by the x axis, with its intensity value in the mRNA population represented by the y axis. In each scatter plot, points that lie close to a 45-degree diagonal line of "identity" represent genes that are expressed at similar levels in the two mRNA populations. The distance that a point lies along this line denotes its level of expression, particularly in its relationship to other genes. The perpendicular distance of a point away from the diagonal line represents the degree to which a gene is differentially expressed between two mRNA populations.

RESULTS

Gene Expression Profiles of D3 ES and D3-Differentiated Cells

To characterize genes that may be associated with the initial developmental decisions and mechanisms directing mammalian embryogenesis, we examined the expression levels of 588 known regulatory genes using mouse Atlas cDNA Expression Arrays hybridized with cDNA probes synthesized from mRNA isolated from both D3 ES cells and their RA-induced differentiated progeny. For this study, we employed conditions that lead to the formation of cells that mainly resemble extraembryonic endoderm when ES cells differentiate (Heath and Smith, 1988; Mummery et al., 1990a; Slager et al., 1993; Wilder et al., 1997). The hybridization results of a typical experiment are shown in Figure 1. Each gene is represented by two parallel spots in order to distinguish between specific hybridization signals and nonspecific background signal. Of the 588 genes, nearly 50% (292 genes) are expressed in D3 ES and/or D3-differentiated cells. Importantly, when different mRNA preparations from D3 ES cells are compared by scatter plot analysis, the profiles and concentration levels of the expressed genes represented in each mRNA population reveal a very tight distribution pattern along the diagonal line of "identity" (Fig. 2). This indicates that cultivation of the cells, mRNA isolation, probe preparations, hybridization conditions, and data analyses are highly reproducible. Similar analysis of two mRNA populations isolated from the differentiated progeny arising from the independent differentiation of D3 ES cells by RA also show remarkable reproducibility in the profile and concentration levels of the expressed genes (Fig. 3). Although the distribution pattern is not as "tight" as that observed for the comparisons of the D3 ES mRNA populations, the signal differences for all of the expressed genes within the two D3-differentiated mRNA populations are within 2.5-fold. The looser distribution pattern is likely to reflect slight differences in the makeup of the heterogenous cell populations.

Identification of Differentially Expressed Genes

To identify genes that are differentially expressed when D3 ES cells are induced to differentiate, we compared head-to-head hybridizations of cDNA probes synthesized from mRNA isolated from D3 ES cells, and D3-differentiated cells to Atlas Arrays (see Fig. 1 for the results from such comparison). Scatter plot analysis of the gene expression data presented in the upper and lower panels of Figure 1 (Fig. 4) revealed a much wider distribution pattern than seen in the control hybridizations (compare Fig. 4 with Figs. 2 and 3). Although the majority of the expressed genes lie relatively close to the diagonal line of "identity," many genes exhibit greater than a 2.5-fold change in their steady-state levels. The marked differences in the gene expression profiles between D3 ES cells and their differentiated progeny are corroborated to a considerable degree by a number of genes whose regulated expression in both ES and embryonal carcinoma (EC) model systems have been well characterized by a variety of methods. For the analysis performed in this study, these can be considered "sentinel genes." For example, fibroblast growth factor-4 (FGF-4), which has been shown to be expressed in ES and EC cells but not in their differentiated progeny (Yoshida et al., 1988; Tiesman and Rizzino, 1989; Velcich et al., 1989), is down-regulated approximately 30-fold on the cDNA array, while TGF-β2, whose expression is restricted to the differentiated progeny of ES and EC cells (Kelly et al., 1990; Mummery et al., 1990b; Slager et al., 1991) is up-regulated more than 15-fold (Fig. 4, Tables 1 and 2). These findings argue that the differentiated cell population contained relatively few undifferentiated ES cells. They also argue that the parental cell population contained relatively few differentiated cells.

To enhance the detection of genes that show modest changes in their expression during differentiation (2.5- to 5-fold) and to minimize the number of spuriously identified differentially expressed genes, comparisons between mRNA populations from parental D3 ES cells and their RA-induced differentiated progeny were per-

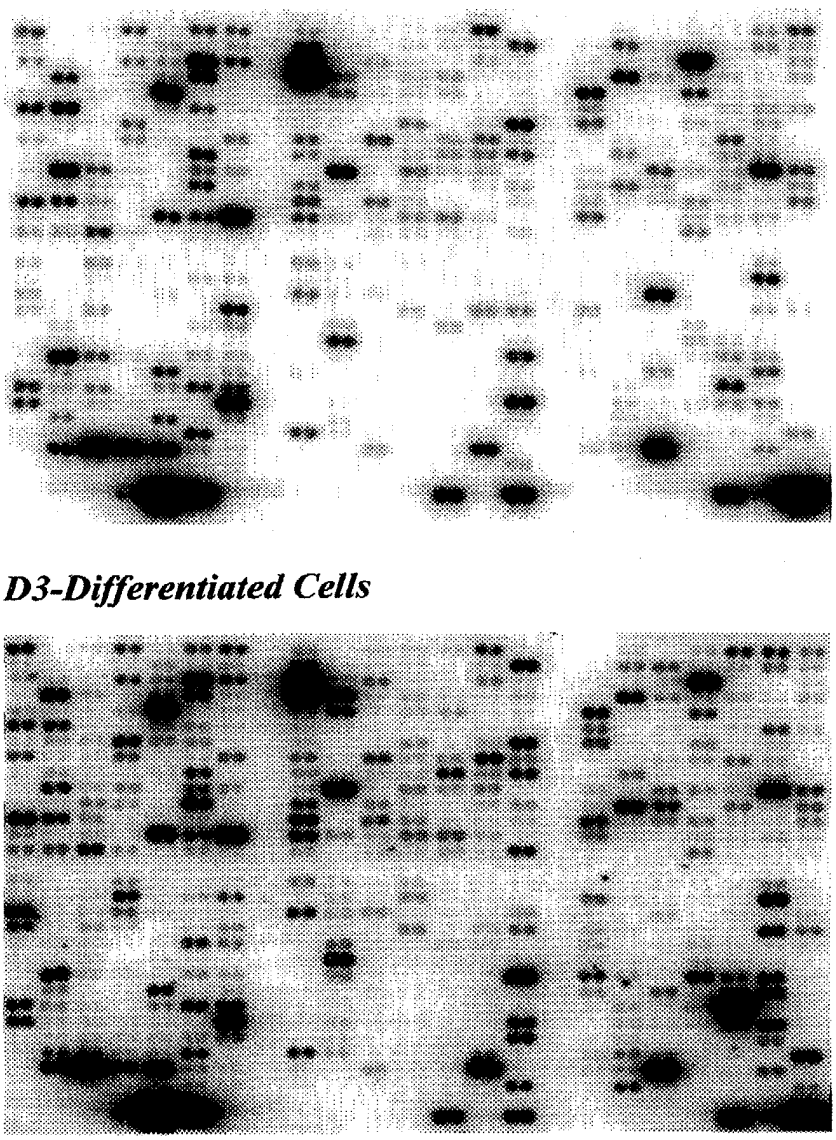

Fig. 1. cDNA array images of the expression pattern of genes in D3 ES cells (top panel) and D3-differentiated cells (bottom panel). Differential hybridization of two identical Atlas Mouse cDNA Expression Arrays was performed as described in Material and Methods. The array contains 588 known regulatory genes divided into six functional groups, with nine putative housekeeping genes located in the bottom row. A complete list of gene names and their locations is available at Clontech's Web page. Comparison between mRNA populations from parental D3 ES cells and their RA-induced differentiated progeny was performed with four different sets of Atlas Array membrane lots with similar results.

formed with four different sets of Atlas Array membrane lots. Only those genes which exhibited an average fold induction or reduction of 2.5-fold or greater across all four membrane lots are listed as differentially expressed. Of the 588 genes on the Atlas Arrays, the steady-state levels of 18 are down-regulated (Table 1) and 61 are up-regulated (Table 2) by a factor of 2.5-fold or greater. These changes in gene expression are highly reproducible and represent changes in the expression of a variety of molecular markers, including: transcription factors, growth factors and their receptors, cytoskeletal and extracellular matrix proteins, cell surface antigens, and intracellular signal transduction modulators and effectors.

DISCUSSION

The mechanisms associated with the initial developmental decisions that occur during embryogenesis are believed to involve the selective modulation of defined sets of genes that facilitate cell differentiation. In this context, we have used cDNA microarray technology to profile the expression of 588 known regulatory genes in D3 ES cells and their RA-induced differentiated progeny for a systematic search of genes whose steady-state

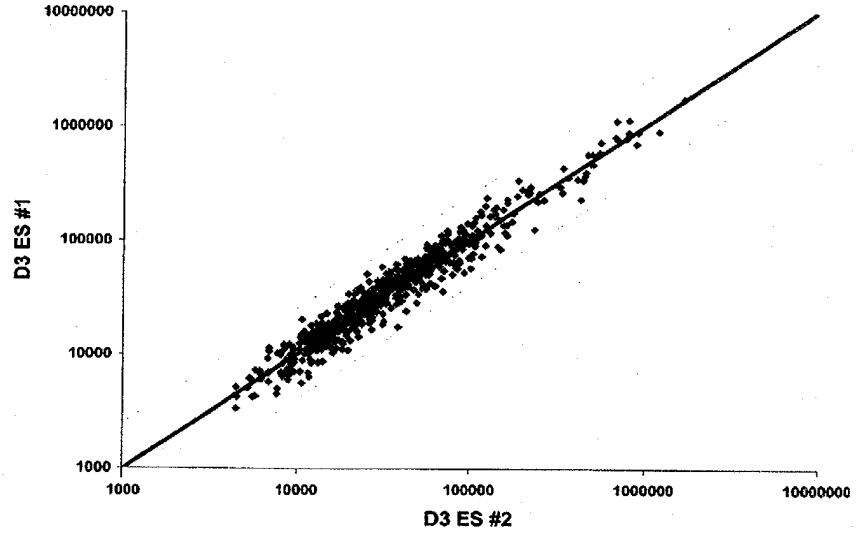

Fig. 2. Scatter plot analysis of log-transformed expression data for two different mRNA populations isolated from D3 ES cells. Each point represents the normalized expression level of an individual gene within both mRNA populations. The predicted "line of identity" is represented by the solid line. The distance that a point lies along this line denotes its level of expression, particularly in relationship to other genes. The perpendicular distance of a point away from the diagonal line represents the degree to which a gene is differentially expressed between the two mRNA populations. The distribution pattern of all points lies within the predicted range of a 2.5-fold or less difference in gene expression (dashed lines).

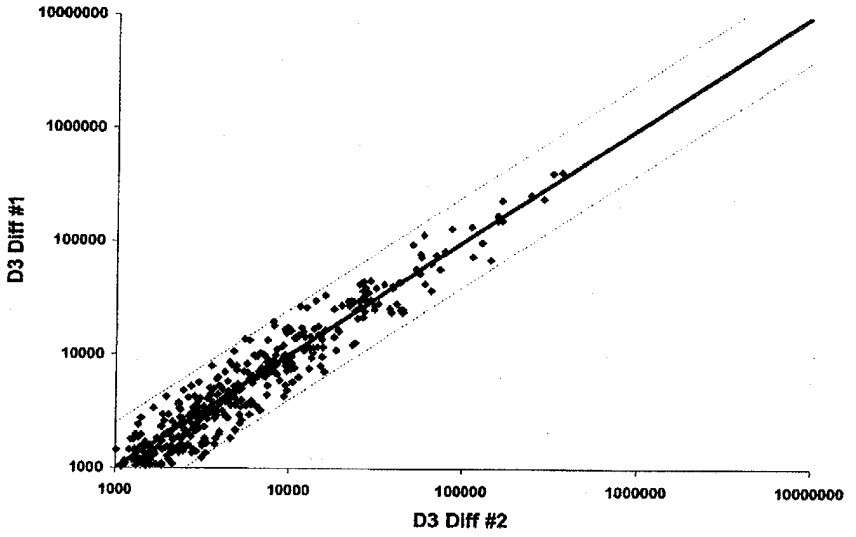

Fig. 3. Scatter plot analysis of log-transformed expression data for two different mRNA populations isolated from RA-induced D3-differentiated cells. Each point represents the normalized expression level of an individual gene within both mRNA populations. The predicted "line of identity" is represented by the solid line. The distribution pattern of all points lies within the predicted range of a 2.5-fold or less difference in gene expression (dashed lines).

levels are modulated during the formation of many of the first distinguishable cell types of the early mammalian embryo, including the extraembryonic endoderm (Evans and Kaufman, 1981; Martin, 1981). We determined that nearly 50% (292) of the arrayed genes are expressed by D3 ES and/or D3-differentiated cells. More importantly, 79 genes were identified whose steady-state levels change by a factor of 2.5-fold or greater in response to RA-induced differentiation. While a number of these genes have been shown previously to be developmentally regulated in embryonal cells and are known to control mammalian development at several different stages, the expression of additional genes (e.g., prothymosin-alpha, PN-1, Spi-2, hsp86, Sox 4, and Cek 5) is shown here for the first time to be differentially regulated at the mRNA level in ES cells and their differentiated progeny. A complete list of the expressed gene and their relative levels of expression is available upon request.

The expression levels of 18 genes decreased during the differentiation of D3 ES cells, including a number of transcription factors (i.e., Oct-3, PEA-3), growth factors (FGF-4), and cell-cycle regulators (cyclins D1 and E). The expression of Oct-3, which encodes a transcription factor that is essential for maintaining the totipotent and pluripotent stem cells of the embryo and the germ line (Brehm et al., 1998; Nichols et al., 1998), was down-regulated approximately 6-fold in response to differentiation. This correlates well with the rapid decrease in Oct-3 transcripts observed by Northern blot

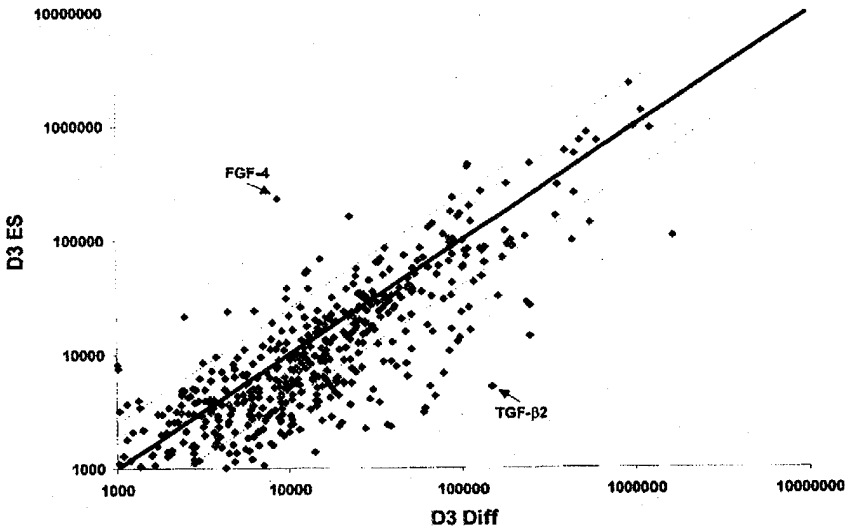

Fig. 4. Scatter plot analysis of log-transformed expression data comparing mRNA populations isolated from D3 ES cells and RA-induced D3-differentiated cells. Each point represents the normalized expression level of an individual gene within both mRNA populations. The predicted "line of identity" is represented by the solid line. Many of the points lie outside the predicted range of 2.5-fold (dashed lines), representing significant changes in the gene expression pattern between the two cell populations. FGF-4 and TGF-β2 are examples of genes whose expression levels change by a factor of more than 15-fold during differentiation.

analysis during the RA-induced differentiation of ES and EC cells (Schöler et al., 1989; Okamoto et al., 1990; Rosner et al., 1990; Minucci et al., 1996; Lamb and Rizzino, unpublished results). In regard to ES cells, the decrease in Oct-3 steady-state levels appears to be a direct response to the transcriptional repression of Oct-3 by RA (Minucci et al., 1996). Not surprisingly, the down-regulation of Oct-3 coincided with a dramatic decrease in the expression of FGF-4 mRNA in ES-differentiated cells (>30-fold). The FGF-4 gene has been shown to be a target of Oct-3 during early mouse development (reviewed in Lamb and Rizzino, 1998). Specifically, FGF-4 transcription requires the binding of Oct-3 (in conjunction with other factors) to an essential distal enhancer located within the 3′ untranslated region of the FGF-4 gene (reviewed in Lamb and Rizzino, 1998). FGF-4 has an essential role during the early stages of mammalian development, as demonstrated by the finding that mouse embryos with both FGF-4 alleles inactivated results in the developmental arrest shortly after implantation (Feldman et al., 1995). Interestingly, through the establishment of FGF-4$^{-/-}$ ES cell lines, it was further demonstrated that FGF-4 appears to serve as an essential paracrine growth factor for the survival and/or proliferation of the differentiated cells derived from the ICM of the developing embryo (Wilder et al., 1997). Hence, using microarray analyses to directly compare the expression pattern of genes for both wild-type and FGF-4$^{-/-}$ ES cells, it may be possible to shed light on the mechanisms by which FGF-4 exerts its effects on the differentiated cells.

Several other developmentally-related genes were down-regulated during the differentiation of ES cells. Expression of PEA-3, a member of the Ets-domain family of transcriptional regulatory proteins thought to be involved in regulating the proliferative capacity of cells (Trimble et al., 1993; Chen et al., 1996; Benz et al.,

TABLE 1. Genes That Are Down-Regulated During the RA-Induced Differentiation of D3 ES Cells

Gene	Average fold reduction[a]	Genbank Accession no.
Transcription factors and general DNA-binding proteins		
TTF-1; RNA polymerase I termination factor	10.3 (4.1–14.9)	X83974
PEA-3; Ets-related protein	8.1 (6.9–9.4)	X63190
N-myc proto-oncogene	7.0 (4.8–10.5)	X03919
Transcription factor S-II	6.5 (3.4–8.4)	D00926
Oct-3; octamer binding transcription factor	6.0 (2.4–8.4)	M34381
Gbx-2	5.4 (2.7–9.3)	L39770
LKLF; Kruppel-like factor	5.2 (2.5–8.7)	U25096
B-myb proto-oncogene	4.6 (2.7–7.3)	X70472
GKLF; gut-specific Kruppel-like factor	3.3 (2.7–3.9)	U20344
Growth factors, cytokines, and chemokines		
FGF-4; fibroblast growth factor-4	32.1 (30.5–33.6)	M30642
Cell cycle regulators		
Cyclin E	3.5 (3.1–3.7)	X75888
Prothymosin alpha	3.5 (2.2–6.0)	X56135
Cyclin D1	3.4 (3.1–3.6)	S78355
Cell adhesion molecules		
CD31; (platelet endothelial cell adhesion molecule 1)	3.7 (2.5–5.0)	L06039
Laminin receptor 1	2.8 (2.1–4.2)	J02870
DNA synthesis		
Ung1; uracil-DNA glycosylase	3.3 (2.0–4.6)	X99018
Stress response proteins		
hsp86; heat shock 86 kD protein	2.5 (2.3–2.7)	M36830
Housekeeping genes		
MOD; murine ornithine decarboxylase	3.1 (2.3–4.8)	M10624

[a]Average fold reduction for each gene was calculated from four different comparisons of the gene expression patterns of D3 ES cells versus D3-differentiated cells. Numbers in parentheses reflect the range of values for the four sets of data.

TABLE 2. Genes That Are Up-Regulated During the RA-Induced Differentiation of D3 ES Cells

Gene	Average fold induction[a]	Genbank Accession no.
Transcription factors and general DNA-binding proteins		
Adipocyte differentiation-associated protein	16.5 (4.6–33.9)	L12721
RAR-beta2; retinoic acid receptor-beta2	9.7 (2.2–13.5)	S56660
CRABP-II; cellular retinoic acid binding protein II	9.6 (2.8–19.9)	M35523
Hoxb-5 (Hox-2.1; homeo box protein 2.1)	6.1 (2.4–12.9)	M26283
ERA-1 protein; (Hoxa-1; Hox-1.6)	5.5 (2.5–10.9)	M22115
Sox 4; SRY-box containing gene 4	5.0 (4.0–5.6)	X70298
Hoxb-9 (Hox-2.5)	4.7 (2.9–6.1)	M34857
Hoxb-8 (Hox-2.4)	4.6 (3.5–6.7)	X13721
ATBF1; AT motif-binding factor	4.5 (2.6–7.3)	D26046
c-Jun; transcription factor AP-1 component	4.0 (2.0–5.5)	J04115
ERCC-5 excision repair protein	2.9 (2.3–3.3)	D16306
Growth factors, cytokines, and chemokines		
IGF-2; insulin-like growth factor-2 (somatomedin A)	27.0 (12.4–53.8)	M14951
TGF-β2; transforming growth factor-beta 2	16.7 (6.6–28.7)	X57413
Cek 5 receptor protein tyrosine kinase ligand	16.5 (4.8–24.9)	U12983
BMP-1; bone morphogenetic protein 1	10.3 (3.3–16.7)	L24755
Growth factor, hormone, cytokines, and chemokine receptors		
IRS-1; insulin receptor substrate-1	15.3 (3.8–21.8)	L24563
PDGFRα; platelet-derived growth factor alpha-receptor	9.4 (4.6–19.6)	M84607
Lymphotoxin receptor	8.5 (6.4–12.1)	U29173
CSF-1; colony stimulating factor-1	6.8 (2.7–11.4)	X05010
IGFR-II; insulin-like growth factor receptor II	5.2 (4.6–6.1)	U04710
Vegfr1; vascular endothelial growth factor receptor 1	4.4 (3.0–6.2)	L07297
Cf2r; coagulation factor II (thrombin) receptor	3.7 (2.3–4.4)	L03529
Interleukin-6 receptor beta chain	2.9 (2.2–3.5)	M83336
Cytoskeleton and motility proteins		
Keratin-18	14.8 (14.0–15.3)	M11686
Keratin-19	8.9 (4.4–15.6)	M28698
Vimentin	7.4 (7.0–7.7)	X51438
Keratin-14	6.8 (4.5–11.5)	M13806
Cell surface antigens and cell adhesion molecules		
Desmocollin 2	7.8 (2.5–13.1)	L33779
Integrin beta 7 subunit	5.5 (4.4–7.7)	M95633
N-cadherin	5.1 (2.7–7.0)	M31131
Integrin alpha 3	3.4 (2.6–4.5)	D13867
Dystroglycan 1	3.3 (2.1–3.9)	U43512
P-selectin	3.0 (2.2–4.2)	X91144
Protein turnover (proteases and inhibitors)		
Serine protease inhibitor homolog J6	9.7 (3.2–14.6)	J05609
Collagenase type IV	8.6 (5.5–13.9)	M84324
Tissue plasminogen activator	6.3 (3.9–11.1)	J03520
Cathepsin B	4.9 (4.3–5.6)	M14222
Cathepsin L	4.9 (3.7–6.0)	X06086
PN-1; protease nexin 1	4.5 (3.5–6.5)	X70296
Spi-2; serine protease inhibitor 2	3.3 (2.7–3.9)	M64086
Cathepsin D	3.3 (2.2–4.0)	X53337
TIMP-3; tissue inhibitor of metalloproteinases-3	2.8 (2.5–3.1)	L19622
Intracellular signal transduction modulators and effectors		
Zyxin	7.2 (3.9–8.2)	X99063
p21/Cip1/Waf1; cdk-inhibitor protein 1	4.0 (2.9–4.6)	U09507
Mdk2; mouse developmental kinase 2	3.7 (2.7–4.3)	Z49085
MAPKAPK-2; MAP kinase-activated protein kinase-2	3.7 (2.2–5.6)	X76850
PI3-K p85; phosphatidylinositol 3-kinase regulatory subunit	3.2 (2.3–4.1)	M60651
RAG1 gene activator	2.9 (2.6–3.2)	X96618
Inhibitor of the RNA-activated protein kinase, 58-kDa	2.8 (2.3–3.9)	U28423
Mdm2; p53-regulating protein	2.5 (2.0–3.0)	X58876
SH-PTP2; adaptor protein tyrosine phosphatase	2.4 (2.0–2.6)	D84372
ZO-1; tight junction protein	2.4 (2.0–2.9)	D14340
A-Raf proto-oncogene	2.4 (2.1–3.1)	M13071
Apoptosis proteins		
Clusterin (apolipoprotein J)	10.1 (4.9–14.8)	L08235
TDAG51	8.9 (4.8–16.3)	U44088
Glutathione peroxidase	5.6 (4.2–8.3)	U13705
c-Akt proto-oncogene	4.9 (4.3–5.7)	M94335
Protein tyrosine phosphatase	3.0 (2.5–3.7)	D83966
Stress response proteins		
Cyp1b1; C3H cytochrome P450	9.0 (2.3–24.4)	X78445
MDR1; multidrug resistance protein	2.9 (2.5–3.6)	M14757
Osp94; osmotic stress protein	2.5 (2.1–2.8)	D49482

[a]Average fold induction for each gene was calculated from four different comparisons of the gene expression patterns of D3 ES cells versus D3-differentiated cells. Numbers in parentheses reflect the range of values for the four sets of data.

1997; Taylor et al., 1997) and in cell patterning of the early embryo (Chotteau-Lelievre et al., 1997; Brown et al., 1998), decreased by approximately 8-fold. Similar to Oct-3, PEA-3 expression has been shown to be reduced rapidly by RA in EC cells, suggesting a direct role of RA in the repression of the PEA-3 gene during the initial stages of cell differentiation (Pratt et al., 1993). Expression of the proto-oncogene N-myc was also substantially reduced (approximately 7-fold), with differentiation. N-myc has been shown to increase both the rate of DNA-synthesis and the proliferation rate of cells by shortening the G1 phase of the cell cycle (Lutz et al., 1996). Among the many target genes of N-myc is prothymosin-alpha (Lutz et al., 1996; Moll et al., 1996; Ben-Yosef et al., 1998) and mouse ornithine decarboxylase (MOD; Lutz et al., 1996; Ben-Yosef et al., 1998). The expression of both of these genes was also reduced significantly upon ES cell differentiation. Like N-myc, prothymosin-alpha is thought to play an important role in cellular proliferation by shortening the G1 phase of the cell cycle (Wu et al., 1997), possibly by modulating the remodeling of chromatin through its interaction with histone H1 (Gomez-Marquez and Rodriguez, 1998; Karetsou et al., 1998). These findings correlate well with the lengthening of the cell cycle when ES and EC cells undergo differentiation.

We also found several genes believed to play regulatory roles in the G1/S phase transition of the cell cycle, such as the transcription factor B-*myb* and the cyclins D1 and E, to be down-regulated with differentiation. While the decrease in the expression of these genes may be a reflection of the reduction in the proliferative capacity of the differentiated cells, other evidence suggests that these genes may be transcriptionally repressed during the initial events of RA-induced cell differentiation. In this regard, transcription of both B-*myb* and cyclin E is extremely responsive to positive and negative modulators of E2F activity (Lam and Watson, 1993; DeGregori et al., 1995; Lam et al., 1995). Thus, it is of particular significance that in normal human bronchial epithelial cells RA converts E2F into a transcriptional suppressor of both B-*myb* and cyclin E, which in turn causes growth inhibition of the cells (Lee et al., 1998). Moreover, cyclin D1 expression may decrease in response to lower levels of B-Myb, since B-Myb has been shown to directly modulate cyclin D1 gene expression in proliferating fibroblasts (Arsura et al., 1992; Sala and Calabretta, 1992). In a similar vein, B-Myb and cyclin D1 have been shown to activate the gene promoter of the 70 kD heat shock protein (hsp70) through a novel mechanism involving the heat shock element (HSE; Kamano and Klempnauer, 1997). Although hsp70 is not present on the Atlas Arrays, the array does contain the hsp86 gene, which was found to be down-regulated with differentiation. Like hsp70, the hsp86 gene promoter contains six consensus HSE motifs important for its expression (Dale et al., 1997), implying a potential role for B-Myb and cyclin D1 in the regulation of hsp86 in ES cells.

The use of Atlas Arrays also identified 61 genes whose expression increased during the differentiation of D3 ES cells. As might be expected, many of the protein products of these genes have essential roles in modulating the composition of the cytoskeleton and extracellular matrix of the heterogenous cell types that arise during the initial stages of embryogenesis. Vimentin and keratins −18, −19, and −14, all members of the intermediate filament multi-gene family, were upregulated significantly in the D3-differentiated cells. These genes are likely to have key roles in the mechanical stability, motility, and apoptosis of cells (reviewed in Oshima et al., 1996; Evans, 1998). Vimentin has been shown to be expressed primarily in visceral extraembryonic endoderm and in cells of mesenchymal origin (Grover et al., 1983; Evans, 1998), whereas keratin-18 is localized to the trophectodermal cell layer (the first epithelium of the embryo) and in the extraembryonic endodermal tissues including primitive, parietal, and visceral extraembryonic endoderm (Oshima, 1982; Pankov et al., 1994).

There was an increase in the steady-state levels of a number of proteases and protease inhibitors that are likely to serve important roles in normal mouse development by modulating processes such as extracellular matrix deposition and degradation, cellular migration and invasion, as well as the activation of latent forms of growth factors and other proteinases. Cathepsins B, D, and L, all members of the papain family of lysomal proteases, were up-regulated 3- to 5-fold in the D3-differentiated cells. This agrees with previous studies that first detect transcripts for these genes at the time of embryo implantation, localized specifically to the differentiated trophoblast giant cells and spongiotrophoblasts, as well as to the visceral and parietal endoderm (Hamilton et al., 1991; Afonso et al., 1997). These proteins are capable of digesting matrix molecules, including laminin, collagen IV, and fibronectin, and can extracellularly activate other proteases involved in matrix degradation, such as the metalloproteinases (reviewed in Duffy, 1996). Thus, the cathepsins are likely to be important components for the controlled invasion of the uterine stroma at the time of embryo implantation, and in the cell migratory events of morphogenesis. Other proteases that were up-regulated in the differentiated cells were collagenase type IV (metalloprotease) and tissue plasminogen activator (serine protease). Both of these proteases are implicated in the breakdown of basement membrane components during embryo implantation and development (reviewed in Lala and Graham, 1990). Coincidently, the serine protease inhibitors, J6, PN-1, and Spi-2, as well as the metalloproteinase inhibitor, TIMP-3, were induced significantly in the differentiated cells, underscoring the likelihood that the maintenance and developmental remodeling of the extracellular matrix is controlled precisely.

Numerous growth factor and growth factor receptor genes that have been shown to exert essential roles during embryonic growth and development were in-

duced in the D3-differentiated cells. Transcripts for TGF-β2 were only detected after D3 ES cells were induced to differentiate, which correlates well with previous studies that show TGF-β2 to be expressed by the extraembryonic endoderm during the earliest stages of mammalian development (Kelly et al., 1990; Mummery et al., 1990b; Slager et al., 1991). Since TGF-βs exert potent effects on the proliferation, differentiation, and extracellular matrix production of the extraembryonic endoderm (Kelly and Rizzino, 1989), up-regulation of TGF-β2 during the initial stages of embryogenesis is likely to have pronounced effects on developmental gene expression patterns. Similarly, expression of the IGF-2 gene increased approximately 27-fold in the D3-differentiated cells. In mice, targeted disruption of the IGF-2 gene results in a growth-deficiency phenotype in which the mutant mice are roughly 60% the size of their wild-type litter mates at birth (Dechiara et al., 1990). In addition, it has been suggested that secretion of IGF-2 may modulate the rate and extent of terminal differentiation (reviewed in Stewart and Rotwein, 1996). IGFR-II, one of the receptors for IGF-2, was also up-regulated with differentiation. Despite its unproven status as a signaling receptor, IGFR-II is also critical for normal embryonic growth in mice (Lau et al., 1994). Targeted disruption of IGFR-II results in fetal overgrowth and perinatal lethality (Lau et al., 1994). Interestingly, loss of IGFR-II coincides with overaccumulation of circulating levels of IGF-2 (Lau et al., 1994), suggesting that a major role of IGFR-II in the embryo is to limit the biological effects of IGF-2. Loss of IGFR-II may also result in a lower rate of activation of TGF-βs. TGF-βs are secreted as latent molecules that must be activated to exert their negative growth effects. IGFR-II binds the latent form of TGF-β and may in fact be necessary for activation of TGF-β by certain cell-associated proteases (Flaumenhaft et al., 1993).

IRS-1, a substrate for IGFR-I, is also up-regulated in the D3-differentiated cells. Activation of IGFR-I by ligand binding (i.e., IGF-2) causes the rapid tyrosine phosphorylation of IRS-1 and the cytoplasmic assembly of a complex consisting of a variety of proteins that are responsible for the stimulation of diverse downstream signal transduction pathways, including the MAP kinase pathway (reviewed in Leroith et al., 1995). One of the proteins that the activated form of IRS-1 also interacts with is p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), which in turn leads to induction of several biological effects, including the stimulation of glucose transport and mitogenesis (Cheatham and Kahn, 1995; Cheatham et al., 1995). Importantly, we also see an induction of PI3-K p85 in the differentiated cells. Together, these findings suggest that differentiation enhances and possibly activates signaling pathways for insulin-related growth factors.

Finally, several genes encoding for transcription factors were up-regulated in the D3-differentiated progeny. The expression of the AP-1 component, c-Jun, increased in a similar fashion, as previously observed during the differentiation of EC and ES cells (Yang-Yen et al., 1990; Pankov et al., 1994). Besides its ability to positively regulate its own transcription (Yang-Yen et al., 1990), c-Jun has been shown to activate a number of the differentiation marker genes, including keratin 18 (Pankov et al., 1994), vimentin (Rittling et al., 1989), and tissue plasminogen activator (Janulis et al., 1999). Many of the transcription factors that were up-regulated with differentiation are RA-responsive genes. Transcription of RAR-beta 2 has been shown to be directly activated by RA in EC cells (Martin et al., 1990) and ES cells (Shen et al., 1992), whereas up-regulation of CRABP-II mRNA levels by RA is mainly controlled by a posttranscriptional mechanism (MacGregor et al., 1992). Moreover, numerous members of the homeobox (Hox) gene family were induced in the D3-differentiated cells. The initial induction and progressive activation of Hox gene clusters during vertebrate embryogenesis are controlled largely by RA-responsive enhancers (reviewed in Marshall et al., 1996).

In summary, we have demonstrated the utility of cDNA microarrays in analyzing molecular changes during the initial events of RA-induced ES-cell differentiation. For a number of genes that have been shown previously to be developmentally regulated in embryonal cells, the data obtained by this approach are comparable to those of other methods. Equally important, additional genes whose regulated expression has yet to be characterized in ES cells and their differentiated progeny were identified by Atlas Arrays. Moreover, these results begin to establish similarities in the expression patterns of genes of known function with those that are poorly characterized. Arranging genes by similarities in their temporal expression pattern by cluster analysis (Eisen et al., 1998; Iyer et al., 1999) could provide a powerful means for efficiently gaining insights to the functions of genes for which information is not currently available.

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